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Adaptative directional asymmetric shape in *Testudo hermanni hermanni* Gmelin, 1789 (Reptilia: Testudines: Testudinidae)

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Abstract. Asymmetries consist of deviations from perfect symmetry. If the variation is normally distributed around a mean of zero, fluctuating asymmetry is present; when the variation is normally distributed around a mean that is significantly different from zero, it is called directional asymmetry (DA). We analysed DA comparing plastron shape from 42 captive individuals of *Testudo hermanni hermanni* (24 males and 18 females) on which ventral digital pictures were obtained. Nineteen landmarks were digitized on the plastron, excluding exterior marginal scutes (12 pairs of opposing landmarks and 7 unpaired landmarks on the body mid-line). We then based the asymmetry analysis on the DA scores from Procrustes ANOVA; all effects of the model were statistically significant, being DA clearly higher. Asymmetric component was different between sexes being larger in males. DA is considered by authors to be as an adaptative trait related to active control on the up-righting.

Keywords. directional asymmetry, fitness, fluctuating asymmetry, Hermann's tortoise, physical stress, plastron

Introduction

A common and simple type of symmetry in biological structures is bilateral symmetry, when left and right sides are mirror images of each other (Klingenberg et al., 2002). It is classified into two categories: matching symmetry, where pairs are separate structures on the left and right side, and object symmetry, where a single structure is internally symmetric (Klingenberg et al., 2002; Briones and Guiñez, 2008). There are three recognized asymmetries: fluctuating asymmetry (FA) is when variation is normally distributed around a mean of zero; directional asymmetry (DA) is when variation is normally distributed around a mean that is significantly different from zero; and antisymmetry (AA) is when frequency distribution departs from normality in the direction of platykurtosis or bimodality (Pither and Taylor, 2000; Klingenberg et al., 2002; Gabriel Rivera and Claude, 2008). These asymmetry types have different statistical properties as well as distinct biological origins and implications, and moreover they can occur together in the same trait. DA is genetically determined, and is presumably not related to developmental stability (Pither and Taylor, 2000; Carter et al., 2009; Leśniak, 2018).

The tortoise shell is a remarkable evolutionary novelty that defines the order Chelonia. The tortoise shell is found in three general forms based on the nature and degree of ossification: hardshells, softshells, and leatherbacks (Wyneken et al., 2008). This shell is composed of two main parts, the dorsal carapace and the ventral plastron, connected along the midflanks by lateral bridges (Wyneken et al., 2008). Hermann's tortoise *Testudo hermanni* (Family Testudinidae), is a medium-sized terrestrial species (average carapace length ca. 130 to 180 mm), widespread in the European Mediterranean region (Bertolero et al., 2011). Currently two subspecies are distinguished: *T. h. hermanni* in Western Europe and *T. h. boettgeri* in Eastern Europe, the border between them being the Po Valley in northeastern Italy (Bertolero et al., 2011). The species inhabits most Mediterranean vegetation habitats, but is typically found in semi-open formations of stony, sun-drenched hills with low and sparse vegetation and grass (Bertolero et al., 2011). It is under increasing stress due to habitat reduction and fragmentation. At the European level, it is considered vulnerable (European Commission, 1992).

Morphology offers a rich, relatively cost-effective and easily accessible source of relevant data that provide insights into adaptative traits within a group for which there are no direct means of measuring (see for instance

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Mac Call (2014) and Nagle et al. (2018) for some examples on aquatic turtles). Here, we addressed the following questions: (1) what types of asymmetry occur in populations of *T. hermanni*? (2) Does the level of DA vary among genders?

Material and methods

All specimens used in the analyses were captive tortoises without any detectable abnormality, such as injuries by predators, or unusual additional scutes or plaques. Digital images of all specimens (n=42; 24 males and 18 females) were obtained using a Nikon (D5100) digital camera.

On each tortoise's plastron, we digitized 19 landmarks on its ventral aspect, excluding exterior marginal scutes (12 pairs of opposing landmarks and 7 unpaired landmarks on the body mid-line; Figure 1). Most of the landmarks were all optimal (Type I), as their spatial positions are defined on the basis of highly repeatable and unambiguous anatomical locations: sutures of gular, humeral, pectoral, abdominal, femoral and anal scutes. The coordinates of landmarks were recorded using TpsDig software (Rohlf, 2015). All landmarks were digitized twice by the same person (Margalida Cladera) to minimize digitizing errors.

To obtain shape data, landmark configurations were superimposed using the generalized Procrustes method which is based on a generalized least-squares minimization of the distance between corresponding landmarks. As a turtle shell has an internal plane of symmetry, we based our analyses on object symmetry (e.g. possessing an internal line or plane of symmetry). Landmark configurations are compared by this superimposition, which is achieved by translating, rotating and scaling all configurations to a common reference system (the mean) (Adams et al., 2013). Shape in this context is the residual mismatch and irreducible distance among homologous landmarks after the complete Procrustes alignment, and is thus "invariant" to (i.e., it does not possess any information about) translation, rotation and scale (Bookstein, 1991) (Jaramillo, 2011). Following the Procrustes fit, we used PCA to summarize the sample shape variation into fewer components. As a proxy for size we used the centroid size (CS) of the landmark configurations, which corresponds to the squared root of the sum of the squared distances from each landmark to the centroid (Bookstein, 1991; Jaramillo, 2011).

Landmark configurations were superimposed using Procrustes methods to generate an overall best fit and mathematically remove the effects of digitizing position, orientation and scale (Bookstein, 1991). The coordinates of the superimposed landmarks were then used in a Procrustes ANOVA to assess asymmetry of shape, in relation to individuals (symmetric component of variation), body sides (directional asymmetry DA, i.e. greater development of one body size), their interaction (FA), and measurement error. To test the statistical significance on ANOVA effects, we used permutation tests, which exchange shell configurations across individuals and body sides. Each test used 1,000 random permutations of the observations. Because we were interested in asymmetry we performed a nested Procrustes analysis of variance (ANOVA) with individual as the factor and with the side × individual interaction as a nested effect of individual variation (Klingenberg, 2015). Since individual variation introduces pure symmetric variation, the nested effect corresponds to asymmetry, and the remaining terms represent variation due to measurement error. To avoid making the assumption of equal, independent and isotropic variation of landmarks, we used a MANOVA approach to test for DA and FA. In order to infer pattern of covariation among landmarks, we analysed the covariance matrices for individual variation and asymmetric component. Finally, a regression for each component was done using the component as dependent variable and CS (log-transformed) as independent variable.

A further investigation of joint displacements of landmarks was assessed with the use of Principal Component Analysis (PCA) of covariance matrices, examining one at a time the generated PCs for each factor included in the Procrustes ANOVA. PC coefficients were displayed graphically with vectors indicating the displacement of landmarks. MorphoJ (Klingenberg, 2011) software was used for the analysis.

Results

Because the measurement error was much lower than asymmetry, the latter was a highly significant part of the variation. All effects tested by the Procrustes ANOVA for the plastron were statistically significant (Table 1), showing individual variability, DA and FA, although the DA showed a higher contribution (19.7% *versus* 10.9%). Similar results were obtained by analysing the two sexes separately (data not shown). MANOVA showed no significant DA in the sample (Pillai trace=0.093, p=0.771; Table 1), so rejecting a non-isotropic variation at each landmark.

Table 1. Output for Procrustes ANOVA, and MANOVA for plastron asymmetric component. Sums of squares (SS) and mean squares (MS) are in dimensionless units of Procrustes distances. Because the measurement error was much lower than asymmetry, the latter was a highly significant part of the variation. Df = degrees of freedom.

Effect	SS	MS	Df	*F*	*P*
Individual	0.12144410	0.0003036103	400	6.10	<.0001
Directional asymmetry	0.00179129	0.0000895647	20	1.80	0.0189
Fluctuating asymmetry	0.01990401	0.0000497600	400	4.53	<.0001
Error	0.00922622	0.0000109836	480		

Effect	Pillai trace	*P*
Directional asymmetry	0.93	0.7713
Fluctuating asymmetry	13.15	<.0001

Asymmetric component showed no regression with size (p=0.476, 1.8% of the shape change explained by size change), being significantly larger in males (p=0.019, Procrustes distance=0.011). The comparisons of the covariance matrices for asymmetric variation among individuals was not statistically significant (p=0.966), indicating different patterns of joint displacements of landmarks.

PCA of covariance matrices showed that variation in symmetric component was dispersed in many dimensions. The first four PCs accounted for 73.1% of the total observed variation (Table 2). The same was true for asymmetric component (4 first PCs were needed to account for a 75.3% of the total observed variance) but for a more immediate comprehension of the general tendency of landmarks displacement. In particular, we found that for individual variability the exterior landmarks in the anterior part of the plastron (gular scutes) tended to move towards right, with a similar tendency in the posterior part of the plastron (anal scutes). There was minor left migrations of middle exterior landmarks. The displacement of inner landmarks were much less involved.

Discussion

The use of geometric morphometrics plus multivariate statistical techniques provides a graphic visualization of the morphological variation of the individuals (Mitteroecker, 2009). In this study we used geometric morphometric approach to investigate the variation of plastron symmetry in *Testudo hermanni hermanni*. Tortoises possess a rigid shell and, as such, the measured level of directional asymmetry can be considered accurate with respect to its influence on locomotor performance (Gabriel Rivera and Claude, 2008). ANOVA and CVA analyses showed significant asymmetries. These variations were not due to size differences.

We have demonstrated that symmetric variations play a major role in determining plastron shape among *T. hermanni*. The tortoises used in the present study were previously bred in captivity and then released in semi-natural habitats, where they had to acclimate to new environmental conditions. These factors could cause stress to animals, supporting the hypothesis that part of observed asymmetry could be interpreted as a reaction to non-optimal environmental conditions, as detected in other species of the same group (Gabriel Rivera andClaude, 2008; Băncilă et al., 2012; Zimm et al., 2017). The presence of both FA and DA was an unanticipated result in the current study but according to Van Valen (1962; in (McCall, 2014) multiple asymmetries can exist at the same time. Over half of the symmetric component of shape variation was associated with the first four PCs, with a low variation between them indicating that there is no external pressure determining shape change (high sexual dimorphism or allometry). The differences between our FA results and those of other studies could be due to species or historic site differences. In contrast to FA, the data showed a stronger DA. DA occurs when there is a propensity for one side of a trait to develop more than the other, e.g. there is a handed bias for one side to be larger than the other (Singleton, 2015; Leśniak, 2018). This observation could be assumed to be an adaptive response, expressed as small morphological changes or as FA, which is present but in a lower degree than for DA. Directional asymmetry occurs throughout the animal kingdom, and

Table 2. Principal Component Analysis of covariance matrices for symmetric and asymmetric component for first 19 Principal Components (PC).

PC	Symmetric component			Asymmetric component		
	Eigenvalues	% Variance	Cumulative %	Eigenvalues	% Variance	Cumulative %
1	0.0008761	34.52	34.52	0.0001650	28.81	28.81
2	0.0003853	15.18	49.71	0.0001217	21.25	50.07
3	0.0003620	14.26	63.97	0.0000911	15.91	65.98
4	0.0002326	9.16	73.14	0.0000535	9.35	75.33
5	0.0002187	8.61	81.76	0.0000337	5.88	81.22
6	0.0001237	4.87	86.64	0.0000321	5.60	86.83
7	0.0001045	4.12	90.76	0.0000270	4.72	91.55
8	0.0000620	2.44	93.21	0.0000143	2.51	94.06
9	0.0000507	1.99	95.21	0.0000079	1.39	95.45
10	0.0000407	1.60	96.81	0.0000062	1.09	96.55
11	0.0000326	1.28	98.10	0.0000050	0.87	97.43
12	0.0000186	0.73	98.83	0.0000044	0.77	98.21
13	0.0000116	0.46	99.29	0.0000032	0.56	98.77
14	0.0000095	0.37	99.67	0.0000028	0.49	99.26
15	0.0000044	0.17	99.84	0.0000024	0.41	99.68
16	0.0000025	0.10	99.95	0.0000013	0.23	99.92
17	0.0000005	0.02	99.97	0.0000002	0.04	99.96
18	0.0000004	0.01	99.99	0.0000001	0.02	99.98
19	0.0000002	0.01	100	0.0000000	0.01	100

a left-right axis has been demonstrated for most major phyla (Klingenberg et al., 2002).

It has been also suggested that shell morphology may be less functionally constrained in terrestrial environments than in aquatic ones among tortoises (Gabriel Rivera and Claude, 2008). At this point, biological explanations of this pattern are purely speculative. However, one possible biological explanation would involve up-righting movement, as the pattern of differently shaped scutes on both sides could have developed if tortoises have a need for a side asymmetry. If animals consistently use the same leg they would tend to fall down to the same side. So in highly domed, terrestrial tortoises with short limbs and necks as *T. hermanni* is, a more asymmetrical scutes related to freeing limbs would represent a better active control on the up-righting, being central scutes constrained towards symmetry. And this adaptation would be explained not because of up-righting due to male to male combats, as females present significative -although smaller- levels of DA, too

DA on this species could have developed either over generations through natural selection. The development within a generation through phenotypically plastic hypertrophy through continued use (i.e. the Blacksmith's arm) is rejected, as no asymmetric change appeared according to size. Changes in morphology of males that engage in male-to-male competition have been observed in many other species of tortoises (G. Rivera and Claude, 2008; McCall, 2014; Malashichev, 2016). But this hypothesis had to be tested through direct observation of mating and combat behaviour in males, as well as motrice behaviour in both sexes. Our conclusions reinforce Gould's conviction that fairly simple epigenetic perturbations often underlie complex morphological evolutionary changes (Godfrey and Sutherland, 1996).

Several studies have demonstrated that sexual selection for low levels of asymmetry can result in symmetric individuals attaining increased reproductive success, which helps to maintain low levels of asymmetry within populations (Rowe et al., 1997). However, it has also been predicted that low levels of asymmetry can be maintained by natural selection for functional reasons, as is the case of DA (Gabriel Rivera and Claude, 2008; Urban et al., 2013). So, a natural selection for a kind of functional efficiency, and not sexual selection for low

asymmetry (i.e. genetic quality), has to be the principal factor constraining the level of plastron asymmetry in Hermann's tortoises. It must exist some kind of physical features of the environment that generate selective pressures that lead to directionally directed morphologies (Hoffmann and Hercus, 2006; Gabriel Rivera and Claude, 2008).

DA in plastron shape among tortoises species may be an important aspect of their macroevolution. Understanding the relationship between directional selection and developmental stability may therefore provide insight into the genetic control of developmental stability, and may help us to better understand the variational properties in this group.

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References

Adams, D.C., Rohlf, F.J., Slice, D.E. (2013): A field comes of age: geometric morphometrics in the 21st century. Hystrix **24**(1): 7–14.

Băncilă, R.I., Plăiaşu, R., Tudor, M., Samoilă, C., Cogălniceanu, D. (2012): Fluctuating Asymmetry in the Eurasian Spur-Thighed Tortoise, *Testudo graeca ibera* Linneaus, 1758 (Testudines: Testudinidae). Chelonian Conservation and Biology **11**(2): 234–239.

Bertolero, A., Cheylan, M., Hailey, A., Livoreil, B., Willemsen, R. (2011): *Testudo hermanni* (Gmelin 1789) – Hermann's Tortoise. Conservation Biology of Freshwater Turtles and Tortoises 059.1-059.20.

Bookstein, F.L. (1991): Morphometric Tools for Landmark Data. Geometry and Biology. (Cambridge University Press, Ed.), Morphometric Tools for Landmark Data: Geometry and Biology. Cambridge.

Briones, C., Guiñez, R. (2008): Una revisión de la asimetría bilateral en bivalvos. Revista de Biología Marina y Oceanografía **43**(1): 1–6.

Carter, A.J.R., Osborne, E., Houle, D. (2009): Heritability of Directional Asymmetry in *Drosophila melanogaster*. International Journal of Evolutionary Biology **1–7**

European Commission. (1992): Council Directive 92/43/ECC on the conservation of natural habitats and of wild fauna and flora. Official Journal of the European Union **94**.

Godfrey, L.R., Sutherland, M.R. (1996): Paradox of peramorphic paedomorphosis: Heterochrony and human evolution. American Journal of Physical Anthropology **99**(1): 17–42.

Hoffmann, A.A., Hercus, M.J. (2006): Environmental Stress as an Evolutionary Force. BioScience **50**(3): 217.

Jaramillo, N.O. (2011): Morfometría geométrica: principios teóricos y métodos de empleo. In: Fronteras de investigación en enfermedades infecciosas (pp. 69–87). AntioquiaOmar Triana Chávez, Ana María Mejía Jaramillo, Andrés Mauricio Gómez Palacio: Universidad de Antioquia.

Klingenberg, C.P. (2011): MorphoJ: An integrated software package for geometric morphometrics. Molecular Ecology Resources **11**(2): 353–357.

Klingenberg, C.P. (2015): Analyzing Fluctuating Asymmetry with Geometric Morphometrics: Concepts, Methods, and Applications. Symmetry **7**: 843–934.

Klingenberg, C.P., Barluenga, M., Meyer, A. (2002): Shape analysis of symmetric structures: Quantifying variation among individuals and asymmetry. Evolution **56**(10): 1909–1920.

Leśniak, K. (2018): Directional asymmetry of facial and limb traits in horses and ponies. Veterinary Journal **198**(1): 46–51.

Malashichev, Y. (2016): Asymmetry of righting reflexes in sea turtles and its behavioral correlates. Physiology and Behavior **157**: 1–8.

McCall, W.C. (2014): Evaluating bilateral asymmetry of the adult Western Pond Turtle (*Emys marmorata*). California State University, Chico.

Mitteroecker, P. (2009): The developmental basis of variational modularity: Insights from quantitative genetics, morphometrics, and developmental biology. Evolutionary Biology **36**(4): 377–385.

Nagle, R.D., Rowe, C.L., Grant, J., Sebastian, E.R., Martin, B.E. (2018): Abnormal Shell Shapes in Northern Map Turtles of the Juniata River, Abnormal Shell Shapes in Northern Map Turtles of the Juniata River, Pennsylvania. Journal of Herpetology **52**(1): 59–66.

Pither, J.P., Taylor, D. (2000): Directional and fluctuating asymmetry in the black winged damselfly *Calopteryx maculata* (Beauvois; Odonata: Calopterygidae). Canadian Journal of Zoology **78**: 1740–1748.

Rivera, G., Claude, J. (2008): Environmental media and shape asymmetry: a case study on turtle shell. Biological Journal of the Linnean Society **94**: 483–489.

Rivera, G., Claude, J. (2008): Environmental media and shape asymmetry: a case study on turtle shell. Biological Journal of the Linnean Society **94**(1993): 483–489.

Rohlf, F.J. (2015): The tps series of software. Hystrix, the Italian Journal of Mammalogy **26**(1): 9–12.

Rowe, L., Repasky, R.R., Palmer, A.R. (1997): Size-dependent asymmetry: fluctuating asymmetry versus antisymmetry and its relevance to condition-dependent signaling. Evolution **51**: 1401–1408.

Singleton, M. (2015): Functional Geometric Morphometric Analysis of Masticatory System Ontogeny in Papionin Primates. Anatomical Record **298**: 48–63.

Urban, M.C., Bürger, R., Bolnick, D.I. (2013): Asymmetric selection and the evolution of extraordinary defences. Nature Communications **4**.

Wyneken, J., Godfrey, M.H., Bels, V. (2008): Biology of turtles. Chapter 1. New York: CRC Press.

Zimm, R., Bentley, B.P., Wyneken, J., Moustakas-Verho, J.E. (2017): Integrative and Comparative Biology Environmental Causation of Turtle Scute Anomalies in ovo and in silico. Integrative and Comparative Biology **57**(6): 1303–1311.

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